FLEXIBILITY IN GENE COEXPRESSION AT DEVELOPMENTAL AND EVOLUTIONARY TIMESCALES

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ABSTRACT

The explosion of next-generation sequencing technologies has allowed researchers to move from studying single genes to thousands of genes, and thereby to also consider the relationships within gene networks. Like others, we are interested in understanding how developmental and evolutionary forces shape the expression of individual genes, as well as the interactions among genes. In pursuing these questions, we confronted the central challenge that standard approaches fail to control the Type I error and/or have low power in the presence of high dimensionality (i.e., large number of genes) and small sample size, as in many gene expression studies. To overcome these challenges, we used random projection tests and correlation network comparisons to characterize differences in network connectivity and density. We detail central challenges, discuss sample size guidelines, and provide rigorous statistical approaches for exploring coexpression differences with small sample sizes. We apply these approaches in a species known for rapid adaptation – the Trinidadian guppy (*Poecilia reticulata*). Our findings provide evidence for coexpression network differences at developmental and evolutionary timescales and suggest that flexibility in gene coexpression relationships could promote evolvability.

INTRODUCTION

Genes neither act nor evolve in isolation. Rather, genes are members of physically and functionally interacting networks. There is long-standing interest in how the nature of these interactions influences the extent to which gene sequence and expression changes are constrained at developmental and evolutionary timescales, and thereby influence the evolvability of higher-order phenotypes. On the one hand, genes with many interaction partners (i.e., those that occupy central 'hub' positions in a network) may be targets of developmental switches and evolutionary selection because they most strongly influence network output and phenotypic change (Chateigner *et al.*, 2020; Friedman *et al.*, 2020). Alternatively, expression changes in highly connected genes may be constrained by pleiotropic effects imposed by their many connections (Jeong *et al.*, 2001; Hahn & Kern, 2005), and developmental and evolutionary changes may therefore be more prevalent in peripheral genes that have lower connectivity and lower pleiotropic loads (Kim *et al.*, 2007; Mähler *et al.*, 2017). Despite their contrasting predictions, both hypotheses rely on the assumption that connections among genes – and therefore network topology – are stable. If the relationships among genes are instead flexible across developmental and evolutionary timescales, gene network position may not be a good predictor of evolvability.

The role of interactions among genes in evolutionary processes has historically been difficult to test because physical interaction networks were well-characterized in only very few species (e.g. protein networks in yeast (Jeong *et al.*, 2001; Hahn *et al.*, 2004; Jovelin & Phillips, 2009)) and simultaneously surveying expression in large numbers of genes was challenging, if not impossible. The proliferation of next generation sequencing technologies, and specifically RNA-sequencing (hereafter RNAseq), has removed these constraints from a technical perspective. Although network and coexpression analyses of RNAseq datasets remain less common than gene-wise differential expression analyses, studies in this area provide intriguing – albeit conflicting – results. In support of the idea that genes in central network positions are evolutionary constrained, while those in the network periphery are more evolvable, are studies

demonstrating that centrality in coexpression networks is negatively correlated with divergence in gene expression (Warnefors & Kaessmann, 2013; Mähler *et al.*, 2017; Kuo *et al.*, 2023) and sequence evolution (Josephs *et al.*, 2017; Masalia *et al.*, 2017; Harnqvist, 2021), while genes in peripheral positions show greater magnitude expression divergence (Mähler *et al.*, 2017) and signatures of positive selection (Kim *et al.*, 2007; Mähler *et al.*, 2017). In contrast, other studies provide evidence for a bias toward changes in the expression of and selection on genes with high centrality in coexpression networks (Koubkova-Yu *et al.*, 2018; Chateigner *et al.*, 2020; Friedman *et al.*, 2020; Rennison & Peichel, 2022), supporting the alternative hypothesis that central genes better predict phenotypic variation and are therefore targets of selection. Attempts to resolve these conflicts include hypotheses that preferential divergence in central versus peripheral genes may be associated with different types of traits (Warnefors & Kaessmann, 2013; Des Marais *et al.*, 2017) or distinct selective modes and timescales (Luisi *et al.*, 2015), or that intermediate levels of pleiotropy facilitate evolution (Hämälä *et al.*, 2020). These ideas are intriguing, yet also rely on the assumption that connections among genes are stable rather than flexible.

If the relationships among genes are instead flexible, then the degree of pleiotropy and its presumed consequences are not fixed, and flexibility in coexpression relationships among genes could reduce pleiotropic load (e.g., (Wang et al., 2010;Pavlicev & Wagner, 2012; Pavličev & Cheverud, 2015). In other words, individual genes may be more able to change in expression and drive phenotypic change if their interactions with other genes can be altered to minimize off-target effects. Conversely, flexibility in coexpression relationships might improve the ability of underlying gene expression networks to buffer higher-level phenotypes through homeostatic change (e.g., (Fischer *et al.*, 2016; Badyaev, 2018; Hoke *et al.*, 2019)). Importantly, either scenario implies that the relationships among genes may themselves be targets of selection. Alternatively, changes in gene coexpression could represent drift and/or transcriptional noise if these changes do not amount to selectable differences at the network and/or organismal level. While this last scenario is less interesting from an adaptationist perspective, such ‘neutral’ changes may nonetheless

87 have consequences for evolutionary trajectories, for example by giving rise to cryptic variation
88 that is revealed under novel environmental conditions (West-Eberhard, 2003; McGuigan & Sgrò,
89 2009; Paaby & Rockman, 2014). In brief, all the above alternatives highlight that coexpression
90 relationships could vary at developmental and evolutionary timescales with consequences in the
91 short- and long-term.

92 Interest in coexpression analyses is being met by a growing collection of tools for
93 (differential) gene coexpression analysis (Wang *et al.*, 2017; Chowdhury *et al.*, 2020; Tommasini
94 & Fogel, 2023). While these software packages make advanced network analyses accessible,
95 they do not eliminate the statistical limitations of these approaches. These limitations arise
96 primarily from the combination of small sample sizes and high-dimensional data (tens of
97 thousands of genes) emblematic of transcriptomic studies. Sample sizes have increased as
98 sequencing costs have decreased, yet per group sample sizes commonly remain less than ten.
99 Pooling samples across experimental groups or multiple studies can bring the total experimental
100 sample size into the range recommended for network analyses (e.g., N=20 by (Langfelder &
101 Horvath, 2008; Ballouz *et al.*, 2015)); however, this solution is not viable when the goal is to test
102 if experimental groups differ in coexpression structure. Moreover, correlations in expression
103 derived from experimental groups that differ in their underlying transcriptional networks are
104 particularly hard to interpret. This leaves researchers trapped between an experimental rock and
105 hard place.

106 Like others, we are interested in using transcriptomic analyses to understand the biological
107 basis of complex phenotypes, and specifically in exploring changes in individual genes as well as
108 the interactions among genes. To this end, we characterized the effects of genetic background
109 (high-predation versus low-predation populations) and developmental environment (rearing with
110 and without predator chemical cues) on brain gene coexpression in two parallel, independent
111 evolutionary lineages of Trinidadian guppies (*Poecilia reticulata*). In Trinidad, downstream, high-
112 predation fish have repeatedly and independently colonized upstream, low-predation

113 environments (Gilliam *et al.*, 1993; Barson *et al.*, 2009F; Willing *et al.*, 2010a; Fraser *et al.*, 2015),
114 leading to parallel adaptive changes in life-history, morphology, and behavior (Reznick *et al.*,
115 1990, 2001; Endler, 1995; Reznick, 1997; Magurran, 2005a). In other words, each river drainage
116 represents a naturally replicated experiment demonstrating parallel phenotypic adaptation.

117 Prior work has demonstrated that both evolutionary history with predators and
118 developmental experience with predators shape life history (Torres Dowdall *et al.*, 2012),
119 morphology (Torres-Dowdal *et al.*, 2012; Fischer *et al.*, 2013; Ruell *et al.*, 2013; Handelsman *et*
120 *al.*, 2014), physiology (Handelsman *et al.*, 2013; Fischer *et al.*, 2014), and behavior (Huizinga *et*
121 *al.*, 2009; Torres-Dowdall *et al.*, 2012; Fischer *et al.*, 2016b). Here, we extend a prior analysis
122 (Fischer et a. 2021) that characterized developmental plasticity and genetic differences in
123 transcriptome-wide expression levels using animals from different populations reared with and
124 without exposure to predator cues (Figure 1). To address plasticity and divergence in the
125 coexpression patterns in these same data, we first asked whether connectivity patterns among
126 genes differed based on evolved differences between lineages (i.e., river drainages), adaptive
127 differences between high- and low-predation populations of the same lineage, and developmental
128 differences arising from rearing with or without predators. In essence, these comparisons allowed
129 us to test whether changes in gene coexpression are a feature of divergence across timescales
130 (genetic and developmental) and parallel, independent evolutionary events. Following these
131 comparisons, we further investigated whether connectivity influenced a gene's propensity for
132 expression divergence.

133 In pursuing these questions, we confronted the statistical challenges that standard
134 approaches may fail to control Type I error and/or have low power when dimensionality is high
135 (i.e., large number of genes) and sample size is small. From a technical perspective, we present
136 a case study for those interested in (differential) coexpression with small per-group sample sizes.
137 We discuss key challenges, set clear sample size guidelines to control Type I error while
138 maintaining power, and provide rigorous statistical approaches for exploring coexpression

differences even with small sample sizes that can be readily implemented for similar coexpression analyses in other studies. These methods do not replace other, popular approaches but rather complement them to ensure analysis outcomes can be rigorously interpreted. Using these statistical methods, we find evidence for differences in coexpression relationships based on both genetic background and rearing environment, suggesting that changes in the interactions among genes are associated with phenotypic divergence at developmental and evolutionary timescales.

METHODS

Fish collection and rearing

Samples here are the same as those described in (Fischer *et al.*, 2021). Guppies used in this study were second generation lab-born fish from unique family lines established from wild-caught high-predation (HP) and low-predation (LP) populations in the Aripo and Quare river drainages in the Northern Range mountains of Trinidad. At birth, we split second-generation siblings into rearing environments with (pred+) or without (pred-) predator chemical cues, and they remained in these environments until the completion of the experiment (Figure 1) (as in (Fischer *et al.*, 2016b)). Guppies were individually housed in 12:12 hour light cycle and fed a measured food diet once daily. All experimental methods were approved by the Colorado State University Animal Care and Use Committee (Approval #12-3818A).

In brief, each drainage consists of a 2x2 factorial design that distinguishes genetic from developmental effects of predation (Figure 1). Pair-wise comparisons of biological relevance are: (1) HP pred- vs LP pred-, an experiment comparing populations reared in an environment lacking predator cues to identify genetic differences between populations; (2) HP pred+ vs HP pred-, to identify environmentally induced changes mimicking the situation in which high-predation fish colonize low-predation environments, i.e., “ancestral plasticity”; (3) LP pred- vs LP pred+, to identify environmentally induced changes comparable to the situation in which low-predation fish

are washed downstream and a measure of whether ancestral plasticity is maintained in the derived population; (4) HP pred+ vs LP pred+, to identify genetic differences when fish are raised with environmental cues of predation. We also compared the same experimental groups across drainages (e.g. HP pred+ in Aripo drainage vs HP pred+ in Quare drainage) to understand differences associated with parallel, independent evolutionary lineages.

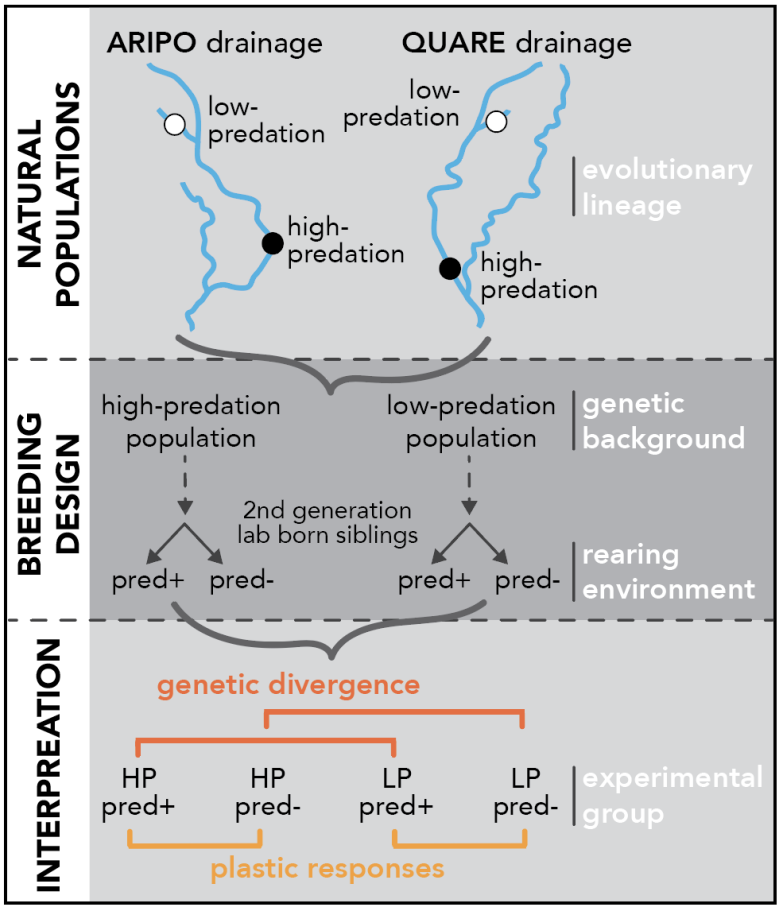

Figure 1. Conceptual overview and interpretation. Top: Natural populations in different river drainages represent distinct evolutionary lineages. Middle: Overview of laboratory breeding design disentangling genetic and environmental effects. Bottom: Interpretation of comparisons of interest between experimental groups resulting from 2x2 breeding design shown above. HP = high-predation, LP = low-predation, pred+ = reared with predator chemical cues, pred- = reared without predator chemical cues. Modified from Fischer et al. 2021.

171 *Tissue collection and processing*

172 We collected brain tissue from mature males in the groups described above 10 minutes after
173 lights on in the morning. We extracted RNA from whole brains using the Qiagen RNeasy Lipid
174 Tissue Mini Kit (Qiagen, Germany) and constructed a sequencing library for each individual using
175 the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Massachusetts,
176 USA). For the Aripo dataset, 40 samples (N=10 per group) were pooled with unique barcodes
177 into eight samples per sequencing library and each library was sequenced on a single lane. For
178 the Quare dataset, 60 samples (N=12-16 per group) were pooled into three sequencing libraries
179 with 20 samples per pool and each library was sequenced in two separate lanes. Libraries were
180 sequenced as 100bp paired-end reads on an Illumina HiSeq 2000 at the Florida State University
181 College of Medicine Translational Science Laboratory (Tallahassee, Florida) in May 2014 (Aripo
182 dataset) and January 2016 (Quare dataset).

183

184 *Differential expression analysis*

185 We reported results of differential expression analyses in another study (Fischer *et al.*,
186 2021). We use normalized values and the resulting differential expression status (differentially
187 expressed (DE) versus non differentially expressed (NDE)) as criterion in analyses performed
188 here (see below). Briefly, we normalized read counts using DESeq2 (Love *et al.*, 2014) and
189 performed differential expression analysis using the lme4 package in R (github.com/lme4). We
190 used generalized linear mixed models with a negative binomial link to accommodate our
191 experimental design and data type. We included family and sampling week as random effects to
192 identify DE genes for the fixed effects of population of origin (HP / LP), rearing environment (pred+
193 / pred-), and their interaction. To label DE and NDE genes, we adjusted p-values for multiple
194 hypothesis testing using a direct approach for FDR control (Storey, 2002) as implemented in the
195 fdrtool package in R (Strimmer, 2008). We considered transcripts differentially expressed (DE) if
196 the adjusted p-value was <0.05, and all other genes non differentially expressed (NDE).

197 Our previous work reported brain gene expression differences based on genetic and
198 developmental influences in both drainages (Fischer *et al.*, 2021). We found that genes exhibiting
199 expression changes in response to rearing differences were also more likely to be differentially
200 expressed between high- and low-predation populations. While this pattern was evident in both
201 river drainages, sets of differentially expressed genes were largely non-overlapping between
202 lineages. For two-sample covariance tests and network comparisons (see below), we categorized
203 genes as DE or NDE based on whether they were differentially expressed for the effect of
204 population of origin within each drainage.
205
206 *Preliminary analyses*
207 Our initial approach was to characterize and explore gene coexpression using the popular
208 Weighted Gene Correlation Network Analysis (WGCNA) package in R (Langfelder & Horvath,
209 2008). We calculated module preservation scores using the methods implements in WGCNA
210 which combine a number of difference preservation statistics to calculate a summary preservation
211 score (Langfelder *et al.*, 2011). We found that, in both drainages, ~50% of gene coexpression
212 modules were not preserved across experimental groups (Supplemental Materials). In other
213 words, we identified substantial differences in network structure between groups, suggesting that
214 the common practice of reconstructing coexpression networks by pooling samples across groups
215 may not be valid. In brief, our preliminary analyses using WGCNA underscored the need for a
216 statistical method to discern network differences across groups when dealing with small group
217 sizes (N=10-12 in our case). We sought to address these issues through the alternative statistical
218 approaches detailed below, and further in the Methods and Supplemental Materials. Our
219 approaches are not intended as a replacement for WGCNA and other similar packages but can
220 be used in conjunction to ensure that WGCNA outputs can be rigorously interpreted.
221
222 *Challenges from small sample, high dimensional data*

In wanting to understand differences in covariance structure and changes in network architecture between our experimental groups, we faced two fundamental challenges. First, controlling Type I error becomes difficult when comparing large networks or covariance structures with limited sample sizes, often leading to spurious discoveries and unreliable results. Also, detecting true effects for separating complicated networks or covariance structures in ultra-high dimensional datasets is difficult due to low statistical power; a common phenomenon in coexpression analysis of RNAseq studies. While there are commonly accepted methods for two sample covariance tests, they either fail to control the Type I error rate with extremely small sample size (e.g., N<25 per group) or have substantially low power (see Supplemental Materials for simulations) (Li & Chen, 2012; Cai *et al.*, 2013; Chang *et al.*, 2017; Yu *et al.*, 2020). Second, comparison of multiple networks is nontrivial, particularly when the networks are of different sizes or have unmatching nodes (Tang *et al.*, 2017; Agterberg *et al.*, 2020; Qi *et al.*, 2024). For gene coexpression analysis these issues apply, for example, when sample sizes vary between groups and are small due to the limitation of experiment constraints, or when comparing subsets of genes of interest that vary in size (e.g., comparing DE vs NDE gene sets, or differently sized coexpression modules) (Agterberg *et al.*, 2020; Alyakin *et al.*, 2024; Jin *et al.*, 2024; Qi *et al.*, 2024).

To highlight these challenges, we first conducted simulation studies using existing high-dimensional methods designed for valid inference on comparing large covariance structures with controlled Type I error rate and reasonable power (Li & Chen, 2012; Cai *et al.*, 2013; Chang *et al.*, 2017; Yu *et al.*, 2020), before implementing our method and examining the real data set (see below). We summarize the outcomes of the simulation studies and exploratory comparisons here and refer the interested reader to additional details provided in the Supplemental Materials.

We found that Type I error rates for existing tests were uncontrolled for small sample sizes of N<50 per group, even when the number of genes was relatively small (250 genes, orders of magnitude smaller than what is typical for RNAseq analysis) (Figure S1). In addition to uncontrolled Type I error, the existing methods were substantially underpowered for small sample

249 sizes. Specifically, the empirical power was overall low (<0.25) for sample sizes N<30 per group
250 (Figure S2). These issues plagued our dataset, which is representative of most RNAseq studies
251 exploring connections between gene expression and behavior (per group samples N<10,
252 ~20,000+ genes). Importantly, these issues are not resolved by subsampling the data to include
253 a smaller number of genes (Figure S3), an approach commonly deployed by network analysis
254 packages (e.g., filtering for the 5,000-8,000 most variable genes in WGCNA or the approach of
255 (Qiu *et al.*, 2021)).
256
257 *Our new high-dimensional covariance comparison*
258 The task of determining whether a common covariance structure can be assumed between two
259 groups for downstream analysis can be addressed using two-sample covariance tests. If this
260 assumption is supported by the data, pooling the samples across groups can improve statistical
261 efficiency in recovering the underlying network and provide a better understanding of its structure.
262 However, from the above simulations, it was clear that existing approaches to compare large
263 covariance structures fail even when using only a small subset of genes. To overcome these
264 issues, we extended the random projection-based covariance test (Wu & Li, 2020) to develop a
265 new two-sample comparison method suitable for our data (see Results and Supplemental
266 Materials).
267 We conduct our newly developed random projection-based tests on residuals from the
268 linear mixed model described above and in (Fischer *et al.*, 2021). We used residuals to remove
269 the mean effects of population of origin and the rearing environment, thereby allowing us to focus
270 on the underlying co-expression patterns among genes, rather than differences in mean
271 expression, when studying dependency structures among genes. We focused on pairwise
272 comparisons of biological interest (Figure 1c and above). Within each drainage, we compared:
273 (1) HP pred- vs LP pred-, (2) HP pred+ vs HP pred-, (3) LP pred- vs LP pred+, and (4) HP pred+
274 vs LP pred+. We also compared the same experimental groups across drainages (e.g. HP pred+

275 in Aripo drainage vs HP pred+ in Quare drainage) to understand differences associated with
276 parallel, independent evolutionary lineages. For both within and between drainage comparison,
277 we considered the four comparisons jointly to control family-wise error rate.
278
279 *Correlation network comparisons*
280 We used correlation network analyses to characterize differences in network structure that
281 accompany the changes in covariance matrices. Because differential expression might drive
282 patterns of differential coexpression patterns, we tested whether DE versus NDE genes differed
283 in their connectivity with other genes and analyzed genetic and developmental influences on
284 network structure separately in DE and NDE gene sets. We used the DE and NDE
285 characterizations determined previously using generalized linear models to detect population
286 differences as summarized above, although we note that the sets of differentially expressed
287 genes were largely non-overlapping between evolutionary lineages (drainages). A challenge with
288 this analysis was the lack of consensus to define networks, in addition to the fact that derived
289 networks will usually have very different sizes and unmatched nodes (i.e., NDE genes far
290 outnumber DE genes, a single gene is inherently in only one category, and the DE genes in the
291 Quare drainage outnumbered those in Aripo).
292 The network comparisons involved two steps: (i) reconstruction of the coexpression
293 network using gene-wise correlations, and (ii) comparing two networks of different sizes. To
294 achieve (i), we first tested whether the correlation between each pair of genes was zero, while
295 controlling the FDR using the method from (Cai & Liu, 2016), as detailed in Supplemental
296 Materials B4.3. Using this method, an undirected edge is drawn between any two genes (nodes)
297 with nonzero correlation, forming the coexpression network. To assess the constructed network's
298 sensitivity to different FDR levels, we compared network summary plots at multiple FDR cutoffs
299 (α = 0.01, 0.05, 0.1). If two networks are distinct, their summary plots will differ (Maugis *et al.*,
300 2017). The network summary plots (Figure S6) suggest that the correlation-based coexpression

network is relatively insensitive to different FDR levels. Therefore, we used a coexpression network with $\alpha = 0.05$ for all subsequent analyses

We compared network pairs of interest to examine their structural differences, using a network summary plot and two-sample network tests based on relative frequencies of different subgraphs adjusted for the sparsity (density of edges) of networks (Maugis et al., 2017; Shao et al., 2022). A major concern in comparing DE versus NDE networks, which has been largely overlooked in literature, is that the different collections of genes in these two sets (e.g., DE genes are a small subset of all genes) make the two corresponding networks have different numbers of unmatchable nodes (Qi *et al.*, 2024). To address this, we adopted the network comparison test proposed by (Shao *et al.*, 2022), which accommodates networks of different sizes by analyzing their network moments for specific motifs using the difference of two subgraph densities adjusted for their edge densities (additional details in Supplemental Materials). Comparing subgraph densities provides a measure of network topology and sparsity that can be visualized using network summary plots. To help readers interpret plots of the real data, we provide a set of example networks and their corresponding network summary plots (Figure 2 and Glossary).

We made various comparisons of the different subgraphs to understand differences based on rearing environment, population of origin, and evolutionary lineage. We conducted one-sided comparisons for each of the three sub-graph motifs (v-shape, triangle, and 3-star). Comparisons fell into four general categories: (1) comparing DE and NDE gene sets within each experimental group, (2) comparing developmental differences (i.e., rearing with (pred+) or without (pred-) predators) for DE and NDE gene sets, (3) comparing populations differences (i.e., high-predation versus low-predation) for DE and NDE gene sets, and (4) comparing experimental groups across evolutionary lineages (i.e., Aripo vs Quare drainage) for DE and NDE gene sets.

325

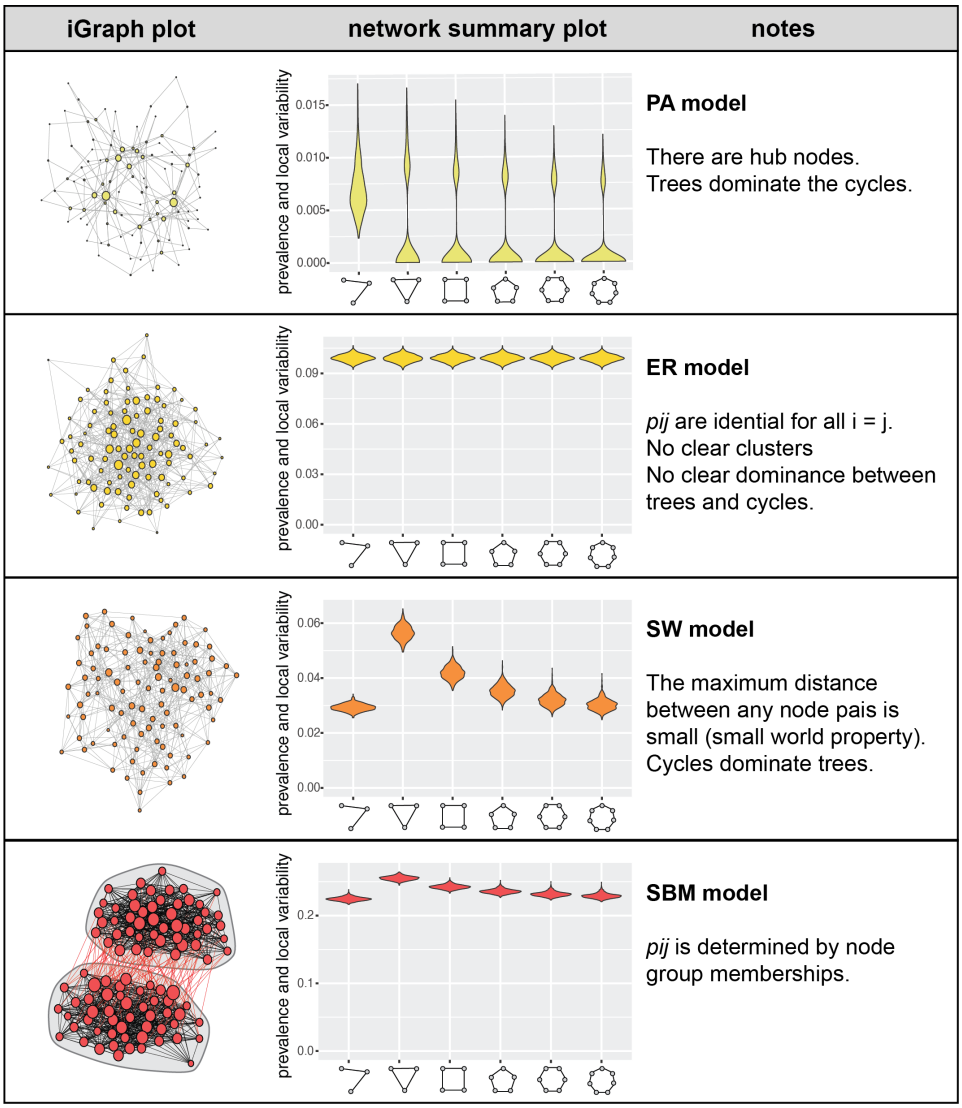

Figure 2. Example network summary plots. Sample networks (iGraphs), their corresponding network summary plots, and a brief interpretation are shown. Examples are intended to help readers unfamiliar with network summary plots interpret summary plots of the real data.

GLOSSARY
adjacency matrix A common means of representing a network using a matrix in which the entries correspond to edges of the network.
graph/network (G) Made up of nodes (*vertices*) and the connections between them (*edges*). Networks can be defined by a vertex set (V) and an edge set (E) as G := (V,E).
network summary plot A plot that provides a scalable and model-free graphical summary of undirected networks. It characterizes network topology and sparsity (see also Figure 2).
random projection A technique used to reduce the dimensionality of a set of points which lie in Euclidean space.
simple network A network that has no self-loops and at most one edge between any pair of vertices.
subgraph A graph formed from a subset of the vertices and edges of a graph G. The subgraph must include all endpoints of the edges in the subgraph. The number of subgraphs can be counted by matrix operations on the adjacency matrix. The *subgraph density* is the proportion of subgraph counts relative to the number of subgraphs in a complete graph in which all pairs of vertices are connected.
undirected network A network in which the edges have no direction, meaning no distinct between in and out.

RESULTS

Changes in coexpression networks based on genetics and environment

We were interested in comparing gene coexpression patterns based on genetic background and rearing environment. To overcome problems associated with limited sample sizes yet high-dimensional data, we used random projection-based tests to compare covariance structures between experimental groups (Wu & Li, 2020). Instead of testing two large covariance matrices directly, these tests compute random projections of the data into lower dimensional spaces and test the equality of variances. Previous work (Wu & Li, 2020) deployed this approach in one-dimensional space, and we developed a generalized version for multi-dimensional space (further

337 details in Supplemental Materials). Using simulations, we confirmed this approach controls type-
338 I error (Figure S4) rate while maintaining power (Figure S5).

339 Applying our method to the real data, we considered the set of all genes that passed
340 filtering criteria (Aripo: 13,446; Quare: 14,379). We found significant differences in covariance
341 structures between high-predation and low-predation fish reared with predators (HP pred+ vs LP
342 pred+) in both drainages (Figure 3 Table 1). Analysis of the Quare dataset found a marginally
343 significant difference between high-predation fish reared with and without predators (HP pred+ vs
344 HP pred-). We also compared the covariance structures between the same treatment groups
345 across drainages. Here, we found significant differences in all comparisons (Table 2). In short,
346 when considering all genes in the daataset, we found evidence for changes in gene coexpression
347 based on evolutionary lineage (drainage), genetic background (population), and rearing
348 environment.

Table 1. Approximated p-values from random projection tests comparing covariance structure for all genes (DE and NDE) between treatment groups.

	ARIPO drainage	**QUARE drainage**
HP pred+ vs HP pred-	1.0000	0.0639
HP pred+ vs LP pred+	0.0149	<0.0001
HP pred- vs LP pred -	1.0000	0.9568
LP pred+ vs LP pred-	0.1258	0.9943

349

Table 2. Approximated p-values from random projection tests comparing covariance structure across drainages.

ARIPO vs QUARE	**p-value**
HP pred-	<0.0001
HP pred+	0.0001
LP pred -	0.0001
LP pred+	0.0107

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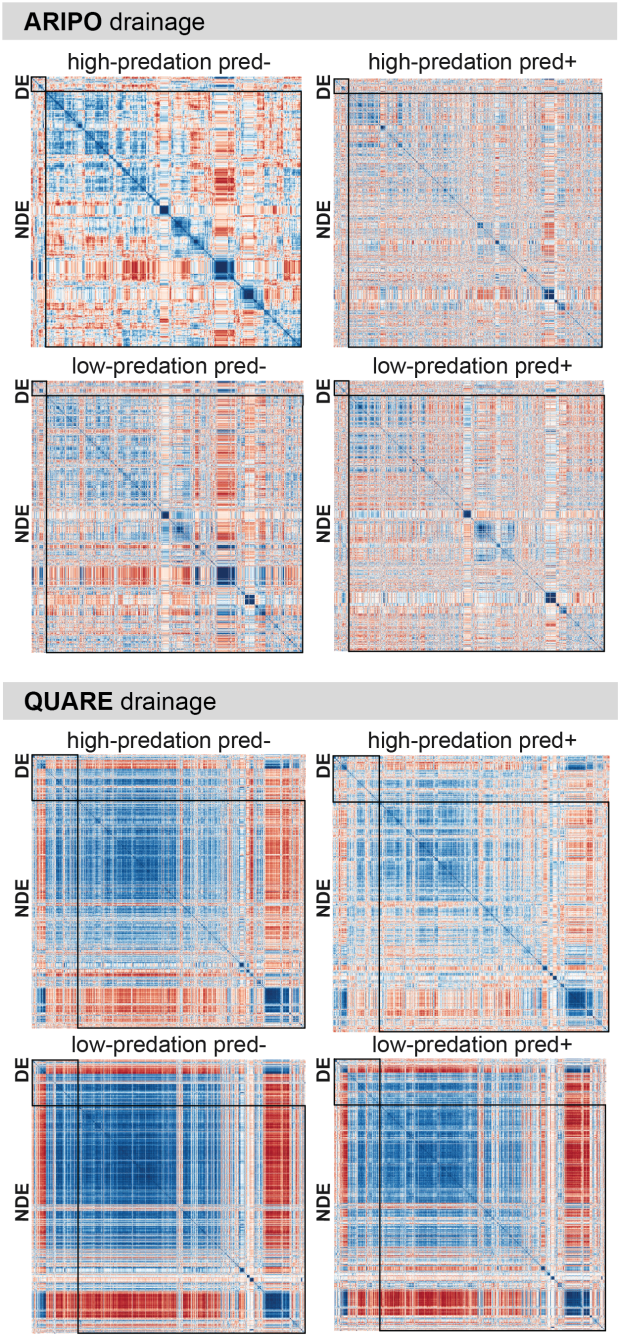


Figure 3. Visualization of coexpression differences between experimental groups and evolutionary lineages. Heatmaps provide a visualization of statistical differences in Pearon's correlations of expression between genes based on genetic background (high- vs low-predation), rearing environment (with (pred+) or without (pred-) predators), and evolutionary lineage (Aripo and Quare drainage). Gene order is determined by hierarchical clustering of the high-predation pred- group, meaning that the same position in two heatmaps represents the correlation of identical pairs of genes. For ease of visualization and computation, only the 1,000 most variable genes are shown.

351 *Coexpression networks and differential expression*

352 To further characterize the changes in coexpression among genes, we compared the prevalence
353 of network motifs across treatment groups. Because we reasoned that divergence in expression
354 levels might itself impact networks, we first addressed whether coexpression networks differed
355 between differentially expressed (DE) and non-differentially expressed (NDE) genes. We
356 performed these comparisons separately for each treatment group, given group-level differences
357 in covariance structures detailed above. Further, because our networks include different gene
358 sets of different sizes, we adopted network comparisons of subgraph densities adjusted for overall
359 edge density (Shao *et al.*, 2022).

360 We compared the v-shape (subgraphs with three nodes and two edges), triangle
361 (subgraphs with three nodes and three edges), and 3-star (subgraphs with four nodes and three
362 edges) prevalence within networks relative the amounts expected given edge densities (see
363 visualizations in Figures 2, 4, and S9). We considered these specific subgraphs commonly used
364 in the network literature as metrics of connectivity and clusterability (Tang *et al.*, 2017; Agterberg
365 *et al.*, 2020; Qi *et al.*, 2024) because all more complex motifs can be deconstructed to these
366 components, and this set of motifs is therefore sufficient to capture network differences such that
367 a greater abundance of complex motifs indicates higher network connectivity and density. Finding
368 different patterns in DE and NDE gene sets within treatment groups (Figure 4, Table S10), we
369 compared network motifs among treatment groups separately in the DE and NDE gene sets.
370 Overall, we found differences in the prevalence of v-shape, triangle, and 3-star motifs in
371 coexpression networks based on genetic and environmental influences, but without a simple rule
372 of directionality (Figure 4, Tables 3 and 4).

373 For groups that differed based on developmental experience, we found pronounced
374 coexpression differences in both drainages but in generally opposite directions: in the Aripo
375 drainage more complex subnetwork motifs were more abundant in fish reared without predator
376 cues while in the Quare drainage complex motifs were more abundant in fish reared with

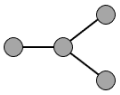
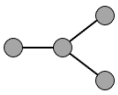
377 predators (Figure 4, Table 3). These patterns were largely concordant for DE and NDE gene sets
378 within both drainages.

379 Population divergence in coexpression networks was also apparent, again with distinct
380 patterns in the two lineages. In the Aripo drainage, high-predation fish from both rearing
381 environments had generally fewer complex network motifs among NDE genes and no differences
382 among DE genes (Figure 4, Table 3). In the Quare drainage, this pattern was flipped, with more
383 complex motifs among NDE genes in high-predation fish, and more complex motifs among DE
384 genes in low-predation fish (Figure 4, Table 3).

385 Differences between the two drainages were also pronounced, with the Aripo drainage
386 having overall fewer complex network motifs, apart from a few differences in the opposing
387 direction for triangles and 3-star motifs among NDE genes (Figure 4, Table 4).

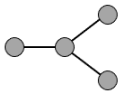
388

Table 3. Network comparisons of treatment groups for DE and NDE genes across datasets. Comparisons of sparsity-adjusted subgraph densities tested the alternatives that gene networks had smaller or larger subgraph density than other networks based on differences in population of origin and rearing environment. P-values from one-sided alternative tests are reported for the v-shape, triangle, and 3-star subgraph types.

		ARIPO drainage			QUARE drainage		
		v-shape	triangle	3-star	v-shape	triangle	3-star
							
	HP < LP						
DE	pred-	0.0003	0.5098	0.3972	<0.0001	<0.0001	0.0088
	pred+	0.7982	0.3333	0.2545	<0.0001	<0.0001	0.1664
NDE	pred-	<0.0001	<0.0001	<0.0001	<0.0001	1	1
	pred+	0.0327	0.3644	0.0021	<0.0001	1	1
	HP > LP						
DE	pred-	0.9997	0.4902	0.6028	1	1	0.9912
	pred+	0.2018	0.6667	0.7455	1	1	0.8336
NDE	pred-	1	1	1	1	<0.0001	<0.0001
	pred+	0.9673	0.6356	0.9979	1	<0.0001	<0.0001
	pred+ < pred-						
DE	HP	0.5407	0.0001	0.0005	<0.0001	1.0000	1.0000
	LP	<0.0001	0.0013	0.0172	<0.0001	1.0000	1.0000
NDE	HP	<0.0001	<0.0001	<0.0001	<0.0001	1	1
	LP	<0.0001	<0.0001	<0.0001	1	1	1
	pred+ > pred-						
DE	HP	0.4593	0.9999	0.9995	1	<0.0001	<0.0001
	LP	1	0.9987	0.9828	1	<0.0001	<0.0001
NDE	HP	1	1	1	1	<0.0001	<0.0001
	LP	1	1	1	<0.0001	<0.0001	<0.0001

390

Table 4. Network comparisons of datasets within treatment groups for DE and NDE genes. Comparisons of sparsity-adjusted subgraph densities tested the alternatives that gene networks for each treatment group in one drainage had smaller or larger subgraph density than networks for the same treatment group in the other drinage. P-values from one-sided alternative tests are reported for the v-shape, triangle, and 3-star subgraph types.

		ARIPO vs QUARE		
		v-shape	triangle	3-star
				
	Aripo < Quare			
DE	HP pred+	<0.0001	<0.0001	<0.0001
	HP pred-	<0.0001	0.0053	<0.0001
	LP pred-	<0.0001	<0.0001	<0.0001
	LP pred+	<0.0001	<0.0001	<0.0001
NDE	HP pred+	<0.0001	<0.0001	<0.0001
	HP pred-	<0.0001	<0.0001	0.9928
	LP pred-	<0.0001	1	1
	LP pred+	<0.0001	<0.0001	0.2933
	Aripo > Quare			
DE	HP pred+	1	1	1
	HP pred-	1	0.9947	1
	LP pred-	1	1	1
	LP pred+	1	1	1
NDE	HP pred+	1	1	1
	HP pred-	1	1	0.0072
	LP pred-	1	<0.0001	<0.0001
	LP pred+	1	1	0.7067

391

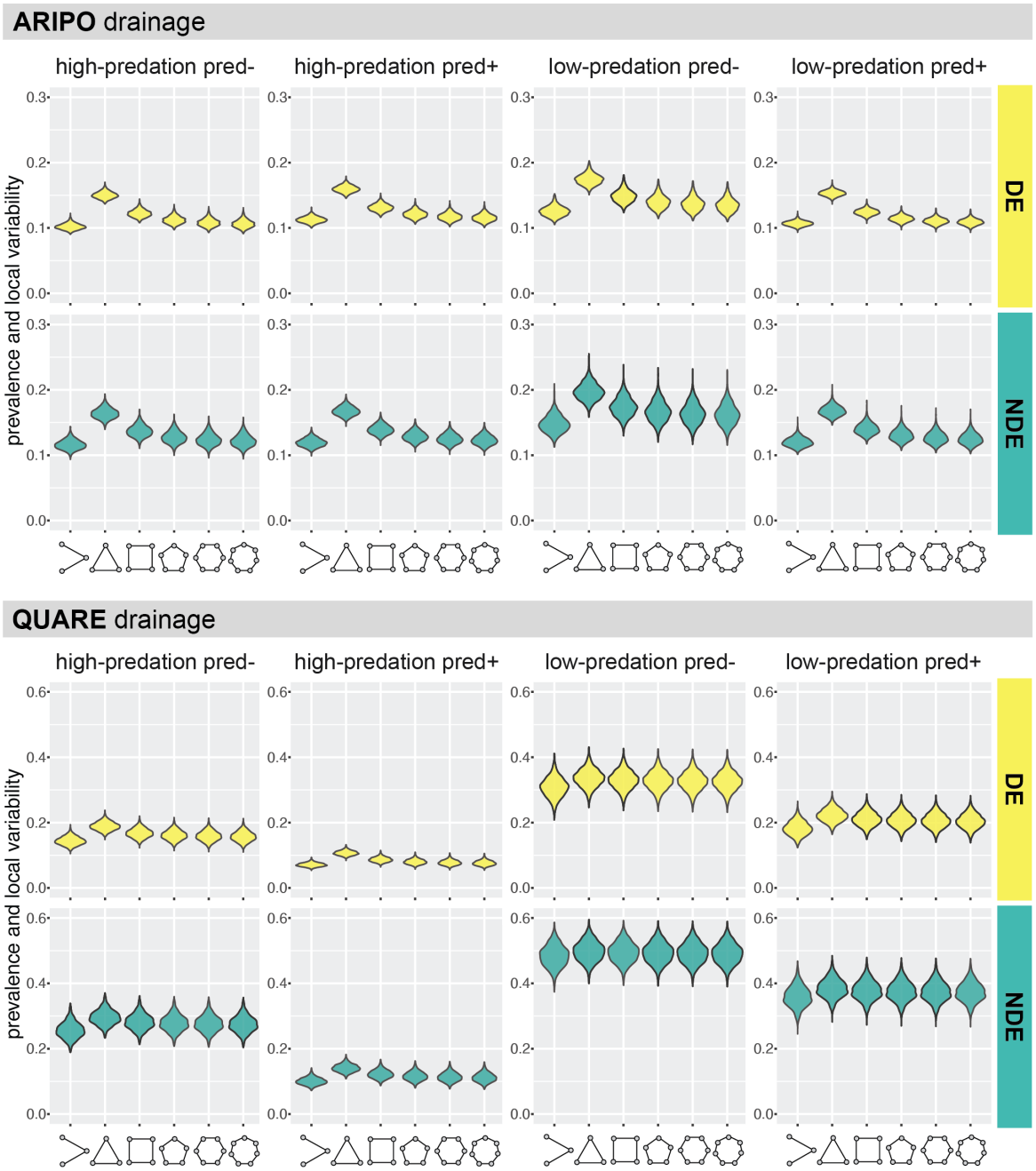


Figure 4. Network summary plots of correlations networks. Plots provide a visualization of network topology and sparsity for networks of DE (yellow) and NDE (teal) genes that differed in mean expression levels between the two populations in each river drainage. Subgraph shapes are depicted on the x-axis. See Tables 3 and 4 for outcomes of statistical comparisons between groups.

392 **DISCUSSION**

393 Our goal in this study was to understand how genetic background and rearing environment shape
394 relationships among genes. We previously characterized expression changes at the level of
395 individual genes (Fischer *et al.*, 2021), and here we were interested in exploring changes in
396 coexpression patterns among genes. Our findings suggest that coexpression patterns are flexible
397 at evolutionary and developmental timescales. Exciting from a biological perspective, these
398 questions present statistical challenges. We discuss the implications of our work from both angles.
399

400 *Overcoming sample size constraints in high-dimensional data*

401 Gene expression studies remain plagued by small per-group samples sizes and high
402 dimensionality. Network construction is far from trivial, if not problematic, under these conditions,
403 especially when network structure – and not just network expression level – differs among
404 experimental groups. In our own study, we had an overall sample size of N=98 individuals, well
405 above the recommendation of N=30 for network construction. However, this total sample size
406 includes samples from two drainages and four experimental groups, and – based on our analyses
407 here and preliminary analyses using the WGCNA package – we found evidence that network
408 structure differs between experimental groups and even more strongly between drainages. These
409 differences are of key biological interest as they suggest that expression relationships among
410 genes (i.e., network structure) are subject to developmental plasticity and evolutionary
411 divergence. However, if network structure differs across experimental groups, then networks must
412 be constructed separately for each experimental group to avoid construction of 'average' networks
413 that can obscure differences of (biological) interest and lead to biased conclusions (Zhao *et al.*,
414 2014; Shojaie, 2021; Li *et al.*, 2022; Sai Li & Li, 2023). To take an extreme example, if two genes
415 have opposing correlations of the same magnitude in two groups, the average correlation across
416 groups will be zero. Thus, it is the per-group sample size that is most important for network
417 construction and comparison when gene coexpression patterns are of interest.

While our per-group sample size of N=10-15 is relatively large for an RNAseq study, it is below the recommended threshold for network construction, such as the minimum sample of N=20 suggested for RNAseq analyses by (Langfelder & Horvath, 2008; Ballouz *et al.*, 2015). As we illustrate, these sample sizes are surprisingly inadequate for recently developed statistical tests thought to be robust against high-dimensionality, to control Type I error, and to maintain power. Indeed, from our simulation experiments, most common methods require N>50 to retain the generally accepted nominal significance levels of 0.05 and satisfactory power exceeding 0.8. Importantly, the potential misinterpretations resulting from these shortcomings are not systematic (i.e., directionally biased) and therefore difficult to predict.

To address the challenges in two-sample covariance testing, we employed a random projection-based test rather than existing methods grounded in large-sample asymptotic theory. As shown in Supplementary Information B, our method effectively controls the type-I error rate and maintains reasonable power across various choices of projection dimensions and numbers of random projections, even when the sample size is as small as N=10. The test results reveal statistically significant differences in the covariance matrices for several group pairs, indicating structural differences in their underlying networks. Our statistical approach uses non–data-driven projections, which offer several advantages: (i) unlike data-driven methods such as PCA or random-skewers or eigentensor decomposition-biased approaches (Wang *et al.* 2019; Hu *et al.* 2025), the random projection-based test naturally preserves the null hypothesis; (ii) in the resulting low-dimensional projected space, powerful and robust test statistics, such as the U-statistics considered in this paper, can be easily constructed; (iii) multiple random projections yield conditionally independent tests, whose aggregation (e.g., via the maximum operator in this work) enhances power. While we demonstrate the utility of this procedure through simulations, a formal theoretical investigation of these advantages is warranted and is left for future statistical research.

As a growing number of studies consider how interactions among genes shape phenotypic differences across timescales, we present our work as a case study to increase awareness of

444 these limitations, present complementary statistical approaches to those commonly used, and in
445 hopes that others will consider these issues in experimental design and analysis.
446
447 *Evidence for genetic and developmental differences in gene coexpression*
448 Using the robust estimation methods we derived, we first identified differences based on
449 both genetic background and developmental environment, with the most pronounced differences
450 in both drainages between high- and low-predation fish reared with predators (HP pred+ vs LP
451 pred+). This comparison represents the ancestral population adapted to life with predators (HP
452 pred+) versus the derived low-predation population adapted to predator-free environments and
453 suddenly re-exposed to predator cues (e.g., as when fish are washed downstream; LP pred+).
454 Fish adapted to a low-predation life are poorly equipped deal with the sudden stressors of
455 predation. Indeed, we previously found HP pred+ fish to be behaviorally least variable and LP
456 pred+ fish to be behaviorally most variable – both in single behaviors and in the correlations
457 among them (Fischer *et al.*, 2016b). In light of findings here, we suggest that disruption of gene
458 coexpression networks could contribute to unpredictable behavioral patterns and correlations.
459 Beyond genetic and developmental differences within each evolutionary lineage, we found
460 that differences were also ubiquitous when comparing between the two lineages, and more
461 evidence for coexpression differences in the Quare as compared to the Aripo drainage. We
462 suggest these patterns arise in part from the extent of genetic divergence between populations:
463 high- and low-predation populations in the Quare drainage show greater genetic (Willing *et al.*,
464 2010a) and gene expression (Fischer *et al.*, 2021) divergence than those in the Aripo drainage,
465 and the two drainages represent distinct evolutionary lineages (Willing *et al.*, 2010a). The
466 importance of genetic background in shaping evolutionary trajectories is highlighted by our
467 previous work demonstrating distinct underlying mechanisms associated with parallel phenotypic
468 adaptation in guppies from distinct evolutionary lineages (Fischer *et al.*, 2021). Similar
469 mechanistic flexibility has also been demonstrated in other systems (Cordero *et al.*, 2018; Jacobs

470 *et al.*, 2020), including those known for parallel phenotypic evolution (e.g. (Laporte *et al.*, 2015;
471 Hanson *et al.*, 2017; Bolnick *et al.*, 2018)). Our findings here extend these observations from the
472 expression of individual genes to coexpression patterns among genes, suggesting that alternative
473 gene expression network configurations can give rise to shared organism-level phenotypes.

474 The prevalence of coexpression differences in our datasets highlights the need to
475 construct coexpression networks individually across experimental groups. If connectivity diverges
476 at evolutionary timescales and shifts with developmental experience, then constructing a single
477 network from pooled samples may confound differential expression differences with differences
478 in connectivity. For example, if a subset of DE genes has higher connectivity in experimental
479 group A versus experimental group B, then connectivity patterns associated with differential gene
480 expression may be obscured when an average network with intermediate connectivity is
481 constructed. Conversely, and non-mutually exclusive, genes differentially expressed between
482 groups A and B could lead to the appearance of strong expression correlation (i.e., strong
483 connectivity) overall, when in fact the within treatment correlations are weak. In brief, pooling
484 samples for network construction when underlying networks in fact differ across experimental
485 groups may obscure precisely the differences researchers are interested in testing.

486 We note that divergence in gene expression and by extension co-expression reflects both
487 adaptive processes as well as non-adaptive and neutral processes (Whitehead & Crawford, 2006;
488 Lynch, 2007). First, low-predation populations in guppies are particularly susceptible to drift,
489 founder effects, and inbreeding depression because they are established by a small number of
490 individuals and experience relaxed predator selection (Magurran, 2005b; Barson *et al.*, 2009;
491 Willing *et al.*, 2010b). Second, whether initial gene expression changes are dominated by adaptive
492 or non-adaptive processes, further expression changes may be homeostatic or compensatory,
493 buffering higher-level phenotypes from evolutionary change, and thereby leading to alternative
494 transcriptional configurations associated with shared organismal phenotypes (e.g., (Abouheif &
495 Wray, 2002; Crawford & Oleksiak, 2007; Fischer *et al.*, 2016b)). Finally, some changes in

496 coexpression may simply reflect transcriptional noise that is filtered out by downstream regulatory
497 processes (e.g., translational regulation). Though certainly present in our dataset, transcriptional
498 noise is an unlikely a major driver of our results as random expression changes would need to be
499 concordant across individuals to be detected as changes in coexpression. At present, we cannot
500 distinguish between adaptive, non-adaptive, homeostatic, and neutral processes. Indeed, a
501 combination of all factors shapes gene expression and coexpression, and distinguishing among
502 alternatives remains a challenge in transcriptomic work. Developing high-dimensional methods
503 such as ours to detect coexpression differences in small sample sizes is an important first step in
504 understanding the causes and consequences of coexpression changes across developmental
505 and evolutionary timescales.
506
507 *Probing links between network coexpression differences, developmental plasticity, and*
508 *evolutionary divergence*
509 By comparing the prevalence subgraph motifs across networks, we were able to make
510 comparisons of network connectivity and clusterability among experimental groups to further
511 characterize the coexpression differences. Our results indicate widespread differences in gene
512 coexpression network structure (both connectivity and clusterability) at developmental and
513 evolutionary timescales; however, these differences do not follow a directional rule. Prior work
514 proposed alternative directional hypotheses, (1) genes in central network positions are
515 evolutionarily constrained and hence less likely to diverge in mean expression levels; and (2)
516 expression changes in genes occupying central hub positions are more likely to have phenotypic
517 consequences and hence more likely to be targets of selection. Rather than supporting either
518 consistently increased or decreased connectivity or clusterability, our findings provide a potential
519 explanation for conflicting evidence surrounding the ongoing debate about whether hub genes
520 are more or less likely to evolve: if coexpression relationships themselves can change both on
521 developmental and short-term evolutionary time scales, then the constraints and/or advantages

522 imposed by high versus low connectivity are not fixed. As a result, the association between
523 network position and differential expression may vary across traits, taxa, and environments and
524 therefore studies.

525 We detected extensive plasticity and divergence in connectivity and clusterability in both
526 DE and NDE gene sets, highlighting that network changes do not have a simple association with
527 differential expression levels of individual genes. We analyzed DE and NDE networks separately
528 with the idea that divergence and plasticity in mean expression levels of DE genes might be
529 driving the covariance differences we report. As a simplest example, covariance between two
530 genes in one experimental group might be disrupted by the molecular or cellular mechanisms that
531 increase or decrease the expression level of one of those genes during population divergence.
532 Our results in Tables 3 and 4 refute the notion that differential gene expression is the primary
533 driver of network differences, as even the genes that lacked detectable differences in mean
534 expression levels (NDE genes) exhibited widespread divergence in coexpression subgraphs. This
535 pattern raises key questions about the sources of coexpression flexibility, prompting future work
536 analyzing the molecular and cellular mechanisms that drive these overall patterns, including
537 changes in transcriptional regulation, mRNA stability, and changing proportions of cell types.

538

539 *Conclusions*

540 Understanding how underlying genetic architecture shapes the maintenance and evolution of
541 complex traits is a fundamental goal of biological research. Over the past two decades, the
542 explosion of next-generation sequencing technologies has allowed us to move beyond the genetic
543 scale – considering one or a few genes or loci – to genomic scales – considering thousands to
544 tens of thousands of genes or loci. Among the key advances afforded by these approaches are
545 the ease of conducting broadscale, exploratory studies; the opportunity to characterize underlying
546 mechanisms in non-model species; and the ability to consider genes in the context of their
547 interactions. As a growing number of studies consider how interactions among genes shape

phenotypic differences across timescales, we provide a case study to increase awareness of limitations and provide suggestions for analysis.

Our findings provide intriguing evidence of extensive coexpression differences at multiple timescales in a species known for rapid adaptation and suggest that flexibility in gene coexpression relationships across time scales may contribute to evolutionary potential. This idea, its generality, and its consequences for adaptation will be revealed by more studies with larger sample sizes and new statistical approaches. We further identify divergence and plasticity in co-expression of genes that do not show differential expression levels, a pattern that raises many questions about the mechanistic basis of coexpression patterns and their divergence. We argue that pooling samples across experimental groups may obscure precisely the differential expression and connectivity differences that we seek to understand. Understanding whether and how relationships among genes change at developmental and evolutionary timescales has consequences for our understanding of how underlying mechanisms shape flexibility and robustness in higher order phenotypes, how animals adapt to novel and changing environments, and how behavior and physiology are regulated in health and disease.

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DATA ACCESSIBILITY

Raw sequencing reads are available through the NCBI SRA repository (PRJNA601479). R code for statistical analyses will be made available on GitHub (https://github.com/EnigmaSong/GeneFlexibilityStudy) upon publication.

AUTHOR CONTRIBUTIONS

EKF and KLH conceived of the study; EKF collected samples and performed molecular work, gene expression mapping, transcript abundance estimation, and preliminary differential expression analyses; YS and WZ devised and performed statistical analyses with input from EKF and KLH; EKF and YS performed data visualization; EKF wrote the manuscript with contributions from all authors.

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